(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 103 564 A1

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 158(3) EPC

(43) Date of publication:
30.05.2001 Bulletin 2001/22

(21) Application number: **99933205.9**

(22) Date of filing: **30.07.1999**

(51) Int. Cl.[7]: **C07K 14/82**, C07K 7/00, C12N 15/00, A61K 39/00

(86) International application number:
PCT/JP99/04130

(87) International publication number:
WO 00/06602 (10.02.2000 Gazette 2000/06)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **31.07.1998 JP 21809398**

(71) Applicant: **Sugiyama, Haruo**
Mino-shi, Osaka 562-0036 (JP)

(72) Inventors:
- **SUGIYAMA, Haruo**
 Mino-shi, Osaka 562-0036 (JP)
- **OKA, Yoshihiro**
 Tondabayashi-shi, Osaka 584-0072 (JP)

(74) Representative: **Maschio, Antonio**
D Young & Co,
21 New Fetter Lane
London EC4A 1DA (GB)

(54) CANCER ANTIGENS BASED ON TUMOR SUPPRESSOR GENE WT1 PRODUCT

(57) A tumor antigen that comprises, as an active ingredient, a product of the Wilms' tumor suppressor gene WT1 or a peptide composed of 7-30 contiguous amino acids containing an anchor amino acid for binding to major histocompatibility complex (MHC) class I in said amino acid sequence, and a vaccine comprising said antigen.

EP 1 103 564 A1

Printed by Xerox (UK) Business Services
2.16.7 (HRS)/3.6

Description

Technical Field

[0001] The present invention relates to tumor antigens based on the products of WT1, the tumor suppressor gene of Wilms tumor. The tumor antigens are useful as anti-cancer vaccines against tumors of the blood such as leukemia, myelodysplastic syndromes, multiple myeloma, and malignant lymphoma, or solid tumors such as gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer, as well as all cancers that express WT1.

Background Art

[0002] The immunological mechanisms to reject foreign material are generally comprised of: the humoral immunity which involves macrophages that recognize antigens and function as antigen presenting cells, helper T cells that activate other T cells etc. by recognizing antigen presented by said macrophages and then producing various cytokines, B cells that differentiates into antibody-producing cells by the action of said lymphokines etc., as well as the cellular immunity in which killer T cells that undergo differentiation in response to antigen presentation, and attack and destroy the target cells.

[0003] At present, cancer immunity is mainly considered to be derived from cellular immunity in which killer T cells participate. In the killer T cell-involved cancer immunity, precursor T cells that recognized tumor antigen presented in the form of a complex between the major histocompatibility complex (MHC) class I and the tumor antigen differentiate and propagate to produce killer T cells, which attack and destroy tumor cells. At this time, tumor cells present, on the surface thereof, the complex of the MHC class I antigen and the tumor antigen, which is targeted by the killer T cells (Cur. Opin. Immunol., 5: 709, 1993; Cur. Opin, Immunol, 5: 719, 1993; Cell, 82: 13, 1995; Immunol. Rev., 146: 167, 1995).

[0004] The above tumor antigen presented by the MHC class I antigen on the surface of the target tumor cells is considered to be a peptide composed of about 8 to 12 amino acids produced after the antigen protein synthesized in the tumor cells underwent processing by intracellular proteases (Cur. Opin. Immunol., 5: 709, 1993; Cur. Opin, Immunol, 5: 719, 1993; Cell, 82: 13, 1995; Immunol. Rev., 146: 167, 1995).

[0005] Currently, antigen proteins are being searched for various cancers, but few have been demonstrated as cancer specific antigens.

[0006] WT1, a Wilms tumor suppressor gene (WT1 gene) was isolated from chromosome 11p13 as one of the causative genes of Wilms tumor based on the analysis of the WAGR syndrome that was complicated by Wilms tumor, aniridia, urogenital anomaly, mental retardation, etc. (Gessler, M. et al., Nature, 343: 774-778 (1990)), and the genomic DNA is about 50 Kb and is composed of ten exons, of which cDNA is about 3 kb. The amino acid sequence deduced from the cDNA is as set forth in SEQ ID NO: 1 (Mol. Cell. Biol., 11: 1707, 1991).

[0007] From the facts that the WT1 gene is highly expressed in human leukemia and that the treatment of leukemia cells with WT1 antisense oligomers results in suppression of cellular growth (Japanese Unexamined Patent Publication (Kokai) No. 9-104627), the WT1 gene has been suggested to promote the growth of leukemia cells. Furthermore, WT1 was found to be highly expressed in solid tumors such as gastric cancer, colon cancer, lung cancer, breast cancer, lung cell cancer, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer (Japanese Patent Application (Tokugan) 9-191635), and the WT1 gene was demonstrated to be a new tumor marker in leukemia and solid tumors. However, it has not been confirmed that the expression products of the WT1 gene are tumor-specific antigens useful as a cancer vaccine.

Disclosure of the Invention

[0008] Thus, the present invention intends to confirm the possibility that the expression product of the WT1 gene is a tumor antigen and to provide a new tumor antigen.

[0009] After intensive research to resolve the above problems, the inventors of the present invention have synthesized polypeptides having 7 to 30 contiguous amino acids containing at least one amino acid that is expected to function as an anchor amino acid in the binding with mouse and human MHC class I and MHC class II in the amino acid sequence of the expression product of the WT1 gene, confirmed that these peptides bind to MHC proteins and, when bound to the MHC class I antigen, induce killer T cells and exert cytocidal effects on the target cell, and thereby have completed the present invention.

[0010] Thus, the present invention provides a tumor antigen comprising an expression product of mouse WT1 or a portion thereof. According to a preferred embodiment, the present invention provides a tumor antigen that comprises, as an active ingredient, a peptide having 6 to 30 amino acids containing an anchor amino acid required for binding to

the MHC molecules in the amino acid sequence as set forth in SEQ ID NO: 1 that corresponds to the cDNA of WT1.

[0011] Furthermore, the present invention provides a tumor antigen that comprises, as an active ingredient, a peptide having 7 to 30 amino acids containing an anchor amino acid for binding to the MHC molecules in the amino acid sequence as set forth in SEQ ID NO: 2 that corresponds to the cDNA of human WT1.

[0012] The present invention also provides a cancer vaccine comprising the above tumor antigen.

Brief Description of Drawings

[0013]

Figure 1 is a graph showing a ratio of $CD4^+$ and $CD8^+$ cells in flow cytometry on the cells immunized with the D^b 126 peptide and non-immunized cells in Example 1.

Figure 2 is a graph that compares the cytocidal effect of the cells immunized with the D^b 126 peptide on the target cells pulsed with D^b 126 peptide and the non-pulsed target cells in Example 2.

Figure 3 is a graph having the same meaning as in Figure 2.

Figure 4 is a graph in which A represents the cytocidal effect of CTL induced by the D^b 126 peptide on the T2 cells pulsed with the D^b 126 peptide in Example 3, and B represents the cytocidal effect of CTL induced by the WH 187 peptide on the T2 cells pulsed with the WH 187 peptide in Example 3.

Figure 5 is a chart showing the result of FACS analysis on the surface markers of CTL induced by the D^b 126 peptide (CD19 cells and CD3 cells).

Figure 6 is a similar chart to Figure 5 with respect to the CD4 cells and the CD8 cells.

Figure 7 is a similar chart to Figure 5 with respect to the CD56 cells.

Figure 8 is a chart showing the result of FACS analysis on the surface markers of CTL induced by the WH 187 peptide (CD19 cells and CD3 cells).

Figure 9 is a chart similar to Figure 8 with respect to the CD4 cells and the CD8 cells.

Figure 10 is a chart similar to Figure 8 with respect to the CD56 cells.

Figure 11 is a graph showing the effect of anti-HLA-A2.1 antibody on the specific lysis of T2 cells pulsed with the D^b 126 peptide by the D^b 126 peptide-specific CTL.

Figure 12 is a graph comparing the lytic activity of the D^b 126 peptide-specific CTL on the target cells expressing or not expressing WT1. In the figure, a shows the result obtained when the E:T ratio is 7.5 : 1 and b shows the result obtained when the E:T ratio is 15 : 1.

Figure 13 is a graph that compares the lytic activity of the D^b 126 peptide-specific CTL on the tumor cells (FBL3) that inherently express WT1 and the tumor cells (RMA) that do not express WT1.

Figure 14 is a graph that compares the lytic activity of the D^b 126 peptide-specific CTL on the cells that were transformed with the WT1 gene and the same cells that were not transformed.

Figure 15 is a graph showing the effect of anti-H-$2D^b$ antibody on the cytotoxicity of the D^b 126 peptide-specific CTL.

Figure 16 is a graph showing the in vivo immunological effect when mice was immunized with the D^b 126 peptide as a vaccine.

Figure 17 is a graph showing the immunological effect when a plasmid expressing WT1 is administered to mice as a DNA vaccine.

Figure 18 is a graph showing the absence of the immunological effect when a plasmid not expressing WT1 is administered.

Best Mode for Carrying Out the Invention

[0014] In accordance with the present invention, K^b and D^b of mouse MHC class I and A* 0201 of human HLA were selected as a basis for designing tumor antigen peptides, and peptides expected to have a high affinity with them were selected.

[0015] Based on the description in Immunogenetics 41: 178-228 (1995), Phe and Tyr at position 5 and Leu and Met at position 8 etc. are expected to be anchor amino acids for binding to K^b, and Asn at position 5 and Met and Ile at position 9 etc. are expected to be anchor amino acids for binding to D^b.

[0016] It is also known that the size of the tumor antigen peptide presented by MHC class I on the surface of tumor cells is about 8 to 12. Therefore, the tumor antigen peptide of the present invention is a peptide having 7 to 30 contiguous amino acids containing an anchor amino acid in the amino acid sequence of the WT1 gene expression product as set forth in SEQ ID NO: 1. The number of amino acids is preferably 8 to 12, for example 8 or 9.

[0017] As a specific embodiment in the present invention, the following peptides comprising 8 amino acids:

K^b 45 Gly Ala Ser Ala Tyr Gly Ser Leu (SEQ ID NO: 3), and
K^b 330 Cys Asn Lys Arg Tyr Phe Lys Leu (SEQ ID NO: 4)

were used as peptides binding to K^b of MHC class I, and the following peptides comprising 9 amino acids:

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5),
D^b 221 Tyr Ser Ser Asp Asn Leu Tyr Gln Met (SEQ ID NO: 6), and
D^b 235 Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 7)

were used as peptides binding to D^b of MHC class I. The underlined amino acids in the above sequences are those amino acids that are expected to function as anchors.

[0018] Then, among these peptides, for K^b 45 and K^b 330 the binding activity with K^b of MHC class I was measured, and for D^b 126, D^b 221 and D^b 235 the binding activity with D^b of MHC class I was measured using the cell line (RMA-S) that does not express the endogenous antigen peptide (empty) and the cell line (RMA-S) that expresses K^b and D^b molecules.

[0019] Thus, RMA-S was cultured at 26°C to effect high expression of MHC class I, and the cultured cells were incubated with the solutions of the test peptides at 37°C for 1 hour. This makes unstable the MHC molecule that does not bind to the peptide leading to their disappearance from the cell surface and leaving MHC class I molecules alone that bound to the peptide. Then using fluorescently labeled monoclonal antibody that recognizes MHC class I (K^b, D^b), RMA-S cells were stained. Finally, the binding dissociation constant was calculated from the average amount of fluorescence per cell (Immunol. Lett., 47: 1, 1995).

[0020] As a result, the following result was obtained:

K^b 45 -4.5784838 (log)
K^b 330 -5.7617732
D^b 126 -6.2834968
D^b 221 -5.7545398
D^b 235 -6.1457624

[0021] As hereinabove stated, both have a strong to moderate binding affinity (Kd value) with K^b or D^b, and the D^b 126 peptide having the highest binding affinity was used in the following experiment.

[0022] For humans also, based on the description in Immunogenetics 41: 178-228 (1995), Leu and Met at position 2 from the N-terminal and Val and Leu at position 9 from he N-terminal are expected to be anchor amino acids for binding to HLA-A* 0201. Thus, the following two peptides having 9 amino acids that meet the above requirement were synthesized in the amino acid sequence of human WT1 protein (Mol. Coll. Biol., 11: 1707-1712, 1991) (SEQ ID NO: 2):

D^b 126; Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5)
(the same as the sequence Of D^b 126 in mouse)
WH 187; Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8)
(anchor amino acids are underlined).

[0023] The binding activity of the above peptides with HLA-A* 0201 was measured as follows:

[0024] The above peptides and T2 cells having the empty HLA-A* 0201 molecules (J. Immunol., 150: 1763, 1993; Blood, 88: 2450, 1996) were incubated at 37°C for 1 hour, and then the T2 cells were stained with fluorescently labeled monoclonal antibody that recognizes HLA-A2.1 to calculate the binding dissociation constant based on the average amount of fluorescence per cell in the FACS analysis.

Binding activity	
Peptide	Kd (M)
D^b 126	1.89×10^{-6}
WH 187	7.61×10^{-6}

[0025] The two peptides have a binding affinity of moderate degree or higher.

[0026] Using the above D^b 126 and WH 187 as a peptide that can combine with human MHC Class I molecules,

the experiment described hereinafter was performed.

[0027] The present invention also relates to a cancer vaccine comprising the above antigen as an active ingredient. This vaccine can be used for prophylaxis or treatment of tumors of the blood such as leukemia, myelodysplastic syndromes, multiple myeloma, and malignant lymphoma, or solid tumors such as gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, and ovarian cancer. The vaccine can be given via oral or parenteral administration such as intraperitoneal, subcutaneous, intradermal, intramuscular, intravenous, and nasal administration.

[0028] As a method of administering the vaccine of the present invention, there can be used a method, in which mononuclear cells are collected from the patient's peripheral blood, from which dendritic cells are removed, and the peptide of the present invention is pulsed thereto, which is then returned to the patient via subcutaneous administration etc.

[0029] Vaccines can contain, in addition to the peptide given as the above active ingredient, pharmaceutically acceptable carriers for example suitable adjuvants such as a mineral gel like aluminum hydroxide; a surfactant such as lysolecithin, pluronic polyol; a polyanions; a peptide; or an oil emulsion. Alternatively, other aggregates that can be mixed into liposomes or blended into polysaccharides and/or vaccines can be used. The dosage is generally 0.1 μg to 1 mg/kg per day.

[0030] In the present invention, DNA encoding the above polypeptide vaccine can also be used as a vaccine (DNA vaccine). Thus, after a nucleic acid encoding WT1 or a portion thereof, preferably DNA, is inserted to a suitable vector, preferably an expression vector, it is administered to an animal to produce cancer immunity. A specific example is shown in Example 9.

Examples

[0031] Then, the following examples will demonstrate that the peptide of the present invention is useful as a tumor antigen or a cancer vaccine.

Example 1.

[0032] One hundred μg of the D^b 126 peptide, 200 μg of porcine lactate dehydrogenase and 0.5 ml of Freund's incomplete adjuvant were intraperitoneally injected to C57BL/6 mice twice every week for immunization treatment. One week after the immunization treatment, the mouse spleen was extracted, from which suspensions of spleen cells were prepared. On the other hand, the irradiated spleen cells of the syngeneic mice pulsed with the D^b 126 peptide were incubated with a solution containing 50 μg/ml peptide at 37°C for 30 minutes, which was used as the antigen presenting cell.

[0033] The above immunized spleen cells and the irradiated spleen cells were co-cultured for 5 days to induce or prepare killer T cells. On the other hand, using the Europium labeled EL-4 cells (expressing K^b and D^b) pulsed with the D^b 126 peptide (incubated at 37°C for 30 minutes with a 100 μg/ml peptide solution) as the target cell in a standard method, a killing assay was performed in the following procedure (Table 1).

[0034] As a result, when the EL-4 cells pulsed with D^b 126 were used as the target, cytocidal effects were observed, but when the EL-4 cells not pulsed with D^b 126 were used as the target, few cytocidal effects were observed.

Table 1

	Mouse A	Mouse B
Peptide +	76.6 %	37.2 %
Peptide -	4.9 %	0.9 %
E/T ratio 40:1		

[0035] Then, the spleen samples that exhibited significant cytocidal effects in the killing assay were stained with the fluorescently labeled anti-CD4 antibody or anti-CD8 antibody, which were then subjected to flow cytometry to analyze the expression of CD4 and CD8.

[0036] As a result, as shown in Figure 1, in the spleen cells immunized with the D^b 126 peptide, there was an increase in the $CD8^+$ cells represented by the killer T cells and the ratio of the $CD8^+$ cells to the $CD4^+$ cells represented by the helper T cells etc. was inversely increased compared to the non-immunized control spleen cells.

Example 2.

[0037] Dendritic cells (DC) derived from the bone marrow of the C57BL/6 mice were prepared in the following manner. According to the standard method, the bone marrow cells were cultured in the presence of GM-CSF to prepare bone marrow-derived dendritic cells (J. Exp. Med. 182: 255, 1995).

[0038] The dendritic cells cultured for 7 days, 10 μM OVAII (Ovalbumin II) and 1 μM D^b 126 peptide were incubated for 3 hours and then washed.

[0039] Then, the above DC cells were intradermally injected to the foot pads and hands of C57BL/6 mice, and on day 5 the lymph nodes were removed to prepare cell suspensions. On the other hand, the B7.1-RMA-S cells (RMA-S cells transfected with a gene encoding B7.1 which is a co-stimulatory molecule) pulsed with D^b 126 and irradiated were prepared.

[0040] Then the above cell suspension derived from the relevant lymph node and the B7.1-RMA-S cells were mixed and cultured for in vitro restimulation.

[0041] Then, on day 5 after the in vitro restimulation, a killing assay was performed using the ^{51}Cr-labeled RMA-S cells as the target. When 1/8 of the total lymphocytes recovered on day 5 after restimulation was used as the effector cell, the E/T ratio was set as the highest one (1.0).

[0042] As shown in Figures 2 and 3, the effector cells derived from the lymph nodes of the mice immunized with the D^b 126 peptide killed the target cells pulsed with said peptide but did not kill the target cells that were not pulsed with said peptide.

[0043] Analysis of the ratio of the $CD4^+$ cells and the $CD8^+$ cells by flow cytometry performed as in Example 1 shows that CD4 : CD8 = 1 : 1.4 to 1.7 and that in the mouse cells immunized with the D^b 126 peptide the $CD8^+$ cells were increased and the ratio of the $CD4^+$ cells : the $CD8^+$ cells (about 2 : 1 in the control cells) was reversed in the cells immunized with the D^b 126 peptide as compared to the non-immunized mouse (control) cells.

Example 3.

[0044] T2 cells (5×10^4) that were irradiated after incubating for 1 hour with the peptide D^b 126 or WH 187 (40 μg/ml) and the peripheral blood mononuclear cells (1×10^6) from a healthy human having HLA-A* 0201 were co-cultured. One week later, T2 cells that were irradiated after incubating for 1 hour with the peptide (20 μg/ml) were added to the above culture system for restimulation. From the following day, human IL-2 (final concentration 100 JRU/ml) was added to the culture.

[0045] Subsequently, after repeating, for five times, stimulation with the T2 cells that were irradiated after being pulsed with the peptide, a killing assay was performed using, as the target, the T2 cells pulsed with the peptide or the T2 cells not pulsed with the peptide. The surface markers of the induced CTL were subjected to FACS analysis.

[0046] The killing assay was performed according to the standard method using, as the target, the Europium-labeled T2 cells pulsed with the peptide.

Effector : Target ratio (E/T ratio) is 10 : 1
Co-cultivation time : 3 hours
The concentration of the peptide in the culture : 5 μg/ml

[0047] The result is shown in Figure 4. A in Figure 4 shows the cytocidal effect of CTL induced using D^b 126 peptide on the T2 cells pulsed with the D^b 126 peptide, and B in Figure 4 shows the cytocidal effect of CTL induced using the WH 187 peptide on the T2 cells pulsed with the WH 187 peptide.

[0048] In either case, more potent cytocidal effects were observed in the T2 cells pulsed with the peptide.

[0049] The results of FACS analysis are shown in Figures 5 to 10. Figures 5 to 7 show the results of human CTL induced with the D^b 126 peptide, indicating that most of the cells were CD8-positive. Figures 8 to 10 show the results of human CTL induced with the WH 187 peptide. The CD4-positive cells and the CD8-positive cells were almost equal in the number.

Example 4.

[0050] In order to test the MHC dependency of the cytolytic activity of the D^b 126 peptide-specific CTL, anti-HLA-A2.1 monoclonal antibody was used to block the cytolytic activity of CTL on the T2 cells pulsed with the peptide. The specific cytolysis of the T2 cells pulsed with the D^b 126 peptide was measured in the presence or absence of monoclonal antibody (BB7.2) that blocks HLA-A2.1 molecule at a E/T ratio of 5 : 1.

[0051] The result is shown in Figure 11. In the figure, the * symbol represents the result obtained using anti-H-$2K^b$ monoclonal antibody in stead of anti-HLA-A2.1 monoclonal antibody. As can be seen from the figure, the addition of 60

μg/ml of anti-HLA-A2.1 monoclonal antibody resulted in the reduction of the cytotoxicity to background of the cytolysis of the T2 cells. Unrelated monoclonal antibody (anti-H-2K^b monoclonal antibody Y3) with the same isotype had no effects on the lysis of the T2 cells.

Example 5.

[0052] It was tested whether the D^b 126 peptide-specific CTL can kill the HLA-A2.1-positive leukemia cells that inherently express WT1. As the target cell, the TF1 cells (express WT-1, HLA-A2.1-positive), the JY cells (do not express WT-1, HLA-A2.1-positive), and the Molt-4 cells (express WT1, HLA-A2.1-negative) were used and cytotoxicity was measured at a E : T ratio of 7.5 : 1 (a) or 15 : 1 (b).

[0053] The result is shown in Figure 12. The D^b 126 peptide-specific CTL exhibited a significant cytotoxicity to the HLA-A2.1-positive leukemia cell TF1 that inherently expresses WT1, but exhibited a cytolysis of a background level to the Molt-4 (which expresses WT1, HLA-A2.1-negative) or the JY cells (which do not express WT1, HLA-A2.1-positive).

Example 6.

[0054] It was tested whether the D^b 126 peptide-specific CTL can recognize tumor cells that inherently express WT1 and can cause cytolysis thereof. Specific lysis was measured at the E/T ratio shown in Figures 13 and 14 for tumor cells (FLB3) that express WT1 and tumor cells (RMA) that do not express WT1 (Figure 13) or for the C1498 cells transfected with the WT1 gene or the C1498 cells not transfected with the WT1 gene (Figure 14).

[0055] As shown in Figure 13, the D^b 126 peptide-specific CTL caused lysis of the FBL3 cells that inherently express WT1 but not the RMA cells that do not express WT1. As shown in Figure 14, the D^b 126 peptide-specific CTL further killed the C1498 cells transfected with the mouse WT1 gene as compared to the parent C1498 cells that do not express WT1. This confirmed that the molecule targeted for cell killing by CTL is indeed the WT1 peptide. These results suggest that the D^b 126 peptide-specific CTL can recognize D^b 126 peptide or the related peptides, which were naturally produced by the intracellular processing of the WT1 protein and presented on the H-2D^b molecules of the WT1-expressing cells.

Example 7.

[0056] In order to test whether the cytolytic activity of CTL is MHC dependent, measurement was performed in the presence of an antibody against the H-2 class I molecule. Thus, cytolytic activity of the D^b 126 peptide-specific CTL against the RMA-S cells pulsed with the D^b 126 peptide was measured in the presence of a titer-adjusted monoclonal antibody against H-2K^b (28.13.3S), H-2D^b (28.11.5S), or H-2L^d (MA143). As the control monoclonal antibody, monoclonal antibody having the same isotype was used.

[0057] The result is shown in Figure 15. Depending on the increased concentrations of antibody against H-2D^b, the lytic activity of CTL against the RMA-S cells pulsed with the D^b 126 peptide was suppressed, whereas antibodies against H-2K^b or H-2L^d did not suppress the lytic activity of CTL. These results indicate that CTL exhibits the cytolysis activity in a H-2D^b-dependent manner.

Example 8.

[0058] It was tested whether in vivo tumor immunity can be elicited by the active immunization with the D^b 126 peptide. Using the LPS-activated spleen cells (solid line in Figure 16) pulsed with the D^b 126 peptide, the LPS-activated spleen cells only (shaded line) or phosphate buffered saline (PBS) only (broken line), mice were immunized once every week. After immunization for 3 weeks, 3×10^7 FBL3 leukemia cells were intraperitoneally administered.

[0059] The result is shown in Figure 16. The mice immunized with the D^b 126 peptide overcame tumor challenge and survived, whereas the non-immunized mice and the mice immunized with the LPS-activated spleen cells could not reject tumor challenge and died. In both of the immunized and non-immunized mice, the presence of ascites was observed three days after the above intraperitoneal injection of tumor cells. Ascites continued to increase in the non-immunized mice, and the mice eventually died. In the immunized mice, on the other hand, ascites started to gradually decrease thereafter, and the mice completely rejected tumor challenge and survived. In the non-immunized mice, natural regression was occasionally observed. The regression is expected to be due to natural induction of CTL specific for Friend leukemia virus (FBL3 leukemia cells are transformed with this virus). Because such CTL induction has occasionally been observed in C57BL/6 mice.

Example 9. DNA vaccine

[0060] One hundred μg of WT1-expressing plasmid DNA (plasmid that continuously expresses WT1 which was prepared by ligating the Sau 3AI fragment of mouse WT1 cDNA (Molecular and Cellular Biology, vol. 11, No. 3, p. 1707-1712 (1991), the left column on p. 1709) to the CMV-IE promoter) (Proc. Natl. Acad. Sci. USA., 92: 11105-11109 (1995)) was intramuscularly injected to 6 to 8 week old C57BL/6 mice every 10 days for a total of three times. Ten days after the last intramuscular injection, mouse spleens were removed to prepare spleen cells. After the spleen cells and mWT1C1498 cells (irradiated with 40 Gy radiation) expressing WT1 were co-cultured at 37°C for 6 days, a killing assay (Europium-labeled) was performed using C1498 (PM5G-mWT1) that expressed WT1 and C1498 (PM5G) that did not express WT1 as the target cell. As used herein, C1498 is a mouse myelogenic leukemia cell line that does not express WT1.

[0061] Cytotoxic T cells (CTL) that kill C1498 (PM5G-mWT1) cells that are expressing WT1 but do not kill C1498 cells (PM5G) that are not expressing WT1 were induced.

[0062] The result is shown in Figure 17.

[0063] As a control, a similar experiment as the above was performed in which plasmid that does not express WT1 (contain no WT1 cDNA) was intramuscularly injected to mice in stead of plasmid that expresses WT1. As in the above experiment, spleen cells were removed. After in vitro stimulation with C1498 (PM5G-mWT1) cells that express WT1, a killing assay was performed.

[0064] As shown in Figure 18, no WT1-specific CTL was induced by intramuscular injection of the control plasmid DNA having no WT1 cDNA.

[0065] The above results demonstrated that the peptide of the present invention indeed functions as a tumor antigen and that it induced the growth of killer T cells (tumor cell-toxic T cells) against tumor cells. Therefore, the tumor antigen peptide of the present invention is useful as a cancer vaccine for leukemia and solid tumors that are accompanied by increased expression of the WT1 gene.

SEQUENCE LISTING

<110>

<120> Cancer Antigen Based on Tumor Suppressor Gene WT1

<130> 983279

<160> 8

<210> 1
<211> 449
<212> PRT
<213> Mouse

<400> 1

```
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Ser
                5                  10                  15
Ser Leu Gly Gly Gly Gly Gly Gly Cys Gly Leu Pro Val Ser Gly Ala
            20                  25                  30
Arg Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala
        35                  40                  45
Tyr Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro
    50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Leu His Phe
                85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Thr Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Ala Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Gln His Ser Phe
                165                 170                 175
```

```
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly Met Ala Ala Gly Ser Ser Ser
                245                 250                 255
Ser Val Lys Trp Thr Glu Gly Gln Ser Asn His Gly Ile Gly Tyr Glu
            260                 265                 270
Ser Glu Asn His Thr Ala Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile
        275                 280                 285
His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Ser
    290                 295                 300
Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys
305                 310                 315                 320
Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys
                325                 330                 335
Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro
            340                 345                 350
Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser Asp
        355                 360                 365
Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln
    370                 375                 380
Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr
385                 390                 395                 400
His Thr Arg Thr His Thr Gly Lys Thr Ser Glu Lys Pro Phe Ser Cys
                405                 410                 415
Arg Trp His Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            420                 425                 430
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu His Val Ala
        435                 440                 445
Leu
449
```

<210> 2

<211> 449

<212> PRT

<213> Human

<400> 2

```
Met Gly Ser Asp Val Arg Asp Leu Asn Ala Leu Leu Pro Ala Val Pro
                  5                  10                  15
Ser Leu Gly Gly Gly Gly Gly Cys Ala Leu Pro Val Ser Gly Ala Ala
             20                  25                  30
Gln Trp Ala Pro Val Leu Asp Phe Ala Pro Pro Gly Ala Ser Ala Tyr
         35                  40                  45
Gly Ser Leu Gly Gly Pro Ala Pro Pro Pro Ala Pro Pro Pro Pro Pro
     50                  55                  60
Pro Pro Pro Pro His Ser Phe Ile Lys Gln Glu Pro Ser Trp Gly Gly
 65                  70                  75                  80
Ala Glu Pro His Glu Glu Gln Cys Leu Ser Ala Phe Thr Val His Phe
                 85                  90                  95
Ser Gly Gln Phe Thr Gly Thr Ala Gly Ala Cys Arg Tyr Gly Pro Phe
            100                 105                 110
Gly Pro Pro Pro Pro Ser Gln Ala Ser Ser Gly Gln Ala Arg Met Phe
        115                 120                 125
Pro Asn Ala Pro Tyr Leu Pro Ser Cys Leu Glu Ser Gln Pro Ala Ile
    130                 135                 140
Arg Asn Gln Gly Tyr Ser Thr Val Thr Phe Asp Gly Thr Pro Ser Tyr
145                 150                 155                 160
Gly His Thr Pro Ser His His Ala Ala Gln Phe Pro Gln His Ser Phe
                165                 170                 175
Lys His Glu Asp Pro Met Gly Gln Gln Gly Ser Leu Gly Glu Gln Gln
            180                 185                 190
Tyr Ser Val Pro Pro Pro Val Tyr Gly Cys His Thr Pro Thr Asp Ser
        195                 200                 205
Cys Thr Gly Ser Gln Ala Leu Leu Leu Arg Thr Pro Tyr Ser Ser Asp
    210                 215                 220
Asn Leu Tyr Gln Met Thr Ser Gln Leu Glu Cys Met Thr Trp Asn Gln
225                 230                 235                 240
Met Asn Leu Gly Ala Thr Leu Lys Gly Val Ala Ala Gly Ser Ser Ser
                245                 250                 255
Ser Val Lys Trp Thr Glu Gly Gln Ser Asn His Ser Thr Gly Tyr Glu
            260                 265                 270
```

```
Ser Asp Asn His Thr Thr Pro Ile Leu Cys Gly Ala Gln Tyr Arg Ile
        275                 280                 285
His Thr His Gly Val Phe Arg Gly Ile Gln Asp Val Arg Arg Val Pro
    290                 295                 300
Gly Val Ala Pro Thr Leu Val Arg Ser Ala Ser Glu Thr Ser Glu Lys
305                 310                 315                 320
Arg Pro Phe Met Cys Ala Tyr Pro Gly Cys Asn Lys Arg Tyr Phe Lys
                325                 330                 335
Leu Ser His Leu Gln Met His Ser Arg Lys His Thr Gly Glu Lys Pro
            340                 345                 350
Tyr Gln Cys Asp Phe Lys Asp Cys Glu Arg Arg Phe Ser Arg Ser Asp
        355                 360                 365
Gln Leu Lys Arg His Gln Arg Arg His Thr Gly Val Lys Pro Phe Gln
    370                 375                 380
Cys Lys Thr Cys Gln Arg Lys Phe Ser Arg Ser Asp His Leu Lys Thr
385                 390                 395                 400
His Thr Arg Thr His Thr Gly Lys Thr Ser Glu Lys Pro Phe Ser Cys
                405                 410                 415
Arg Trp Pro Ser Cys Gln Lys Lys Phe Ala Arg Ser Asp Glu Leu Val
            420                 425                 430
Arg His His Asn Met His Gln Arg Asn Met Thr Lys Leu Gln Leu Ala
        435                 440                 445
Leu
449
```

<210> 3
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide

<400> 3

```
Gly Ala Ser Ala Tyr Gly Ser Leu
 1               5
```

<210> 4
<211> 8

<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide

<400> 4
Cys Asn Lys Arg Tyr Phe Lys Leu
1 5

<210> 5
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide

<400> 5
Arg Met Phe Pro Asn Ala Pro Tyr Leu
1 5

<210> 6
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Peptide

<400> 6
Tyr Ser Ser Asp Asn Leu Tyr Gln Met
1 5

<210> 7
<211> 9
<212> PRT
<213> Artificial Sequence

<220>

<223> Synthetic Peptide

<400> 7

Cys Met Thr Trp Asn Gln Met Asn Leu
1 5

<210> 8

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic Peptide

<400> 8

Ser Leu Gly Glu Gln Gln Tyr Ser Val
1 5

Claims

1. A tumor antigen comprising as an active ingredient a product of the tumor suppressor gene WT1 or a partial peptide thereof.

2. The tumor antigen according to claim 1 that comprises, as an active ingredient, a peptide having 7 to 30 contiguous amino acids containing an anchor amino acid required for binding to the MHC molecule in the amino acid sequence as set forth in SEQ ID NO: 1, or a peptide having 7 to 30 contiguous amino acids containing an anchor amino acid required for binding to the MHC molecule in the amino acid sequence as set forth in SEQ ID NO: 2.

3. The tumor antigen according to claim 1 or 2 wherein said antigen is an antigen of cancers having a high expression of the tumor suppressor gene WT1.

4. The tumor antigen according to claim 1 or 2 wherein said tumor is leukemia, myelodysplastic syndromes, malignant lymphoma, multiple myeloma, gastric cancer, colon cancer, lung cancer, breast cancer, germ cell tumor, hepatic cancer, skin cancer, bladder cancer, prostate cancer, uterine cancer, cervical cancer, or ovarian cancer.

5. The tumor antigen according to any one of claims 1 to 4 wherein said peptide is any one of the following:

K^b 45 Gly Ala Ser Ala Tyr Gly Ser Leu (SEQ ID NO: 3)
K^b 330 Cys Asn Lys Arg Tyr Phe Lys Leu (SEQ ID NO: 4)
D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5)
D^b 221 Tyr Ser Ser Asp Asn Leu Tyr Gln Met (SEQ ID NO: 6)
D^b 235 Cys Met Thr Trp Asn Gln Met Asn Leu (SEQ ID NO: 7), and

WH 187 Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8).

6. The tumor antigen according to claim 5 wherein said peptide is

D^b 126 Arg Met Phe Pro Asn Ala Pro Tyr Leu (SEQ ID NO: 5) or
WH 187 Ser Leu Gly Glu Gln Gln Tyr Ser Val (SEQ ID NO: 8).

7. A cancer vaccine comprising a tumor antigen according to any one of claims 1 to 6.

Fig. 1

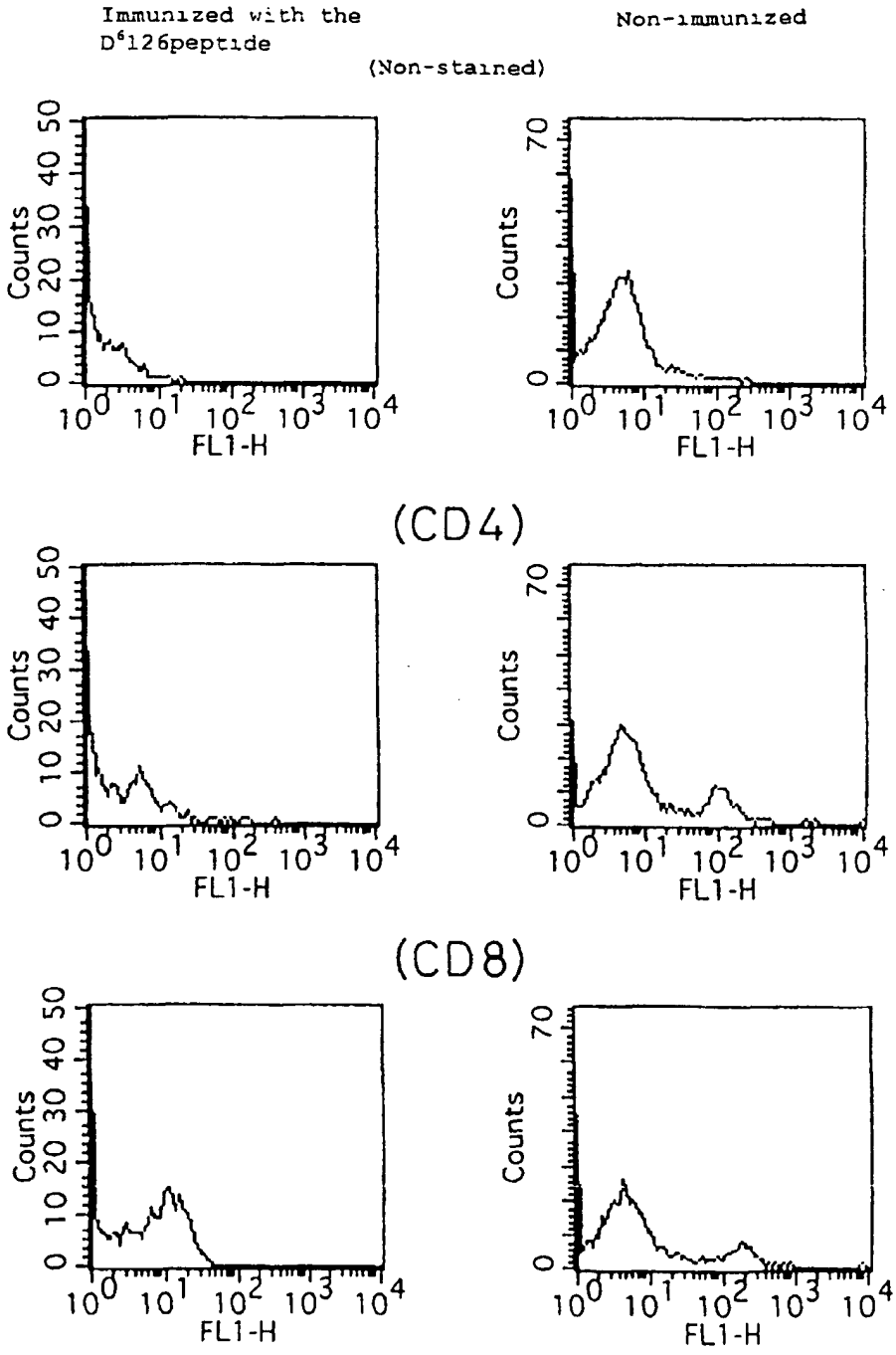

Immunized with the D⁶126peptide
Non-immunized
(Non-stained)
Counts
FL1-H
(CD4)
(CD8)

Fig.2

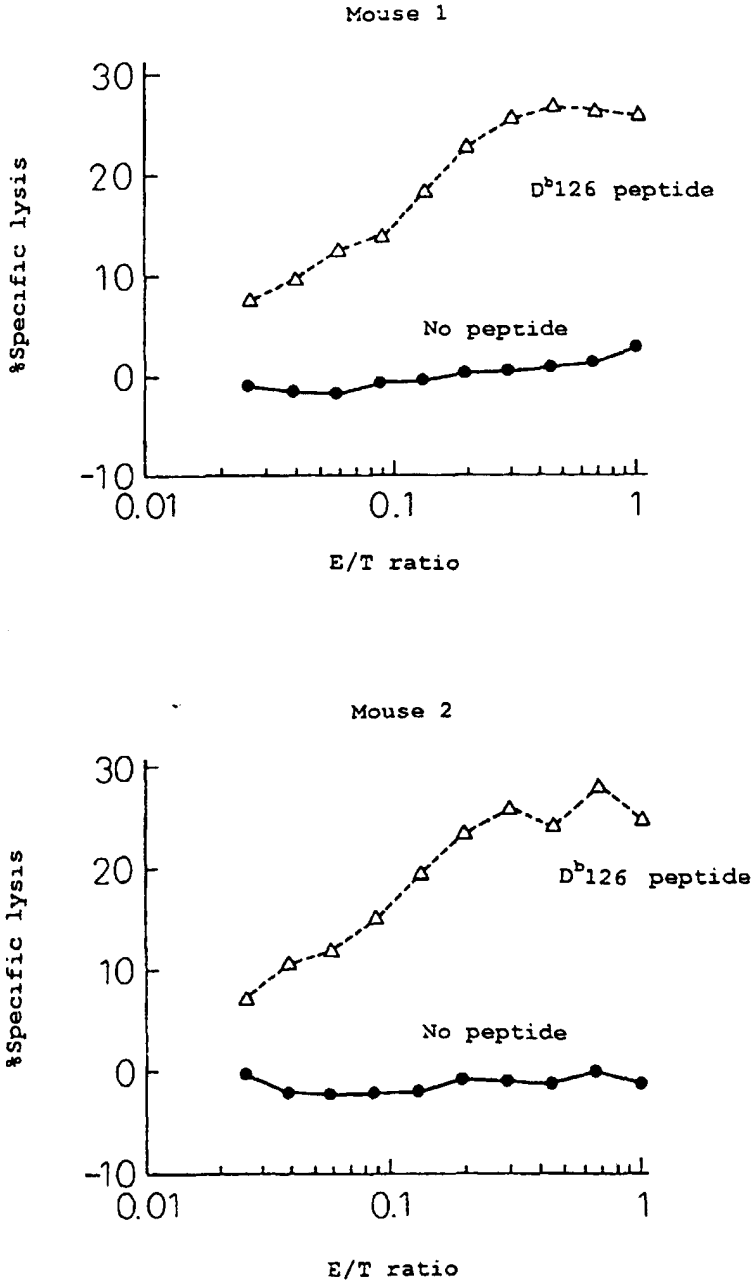

Mouse 1
%Specific lysis
30
20
10
0
-10
0.01
0.1
1
E/T ratio
D^b126 peptide
No peptide
Mouse 2
%Specific lysis
30
20
10
0
-10
0.01
0.1
1
E/T ratio
D^b126 peptide
No peptide

Fig.3


Mouse 3
%Specific lysis
30
20
10
0
-10
0.01
0.1
1
E/T ratio
D^b126 peptide
No peptide

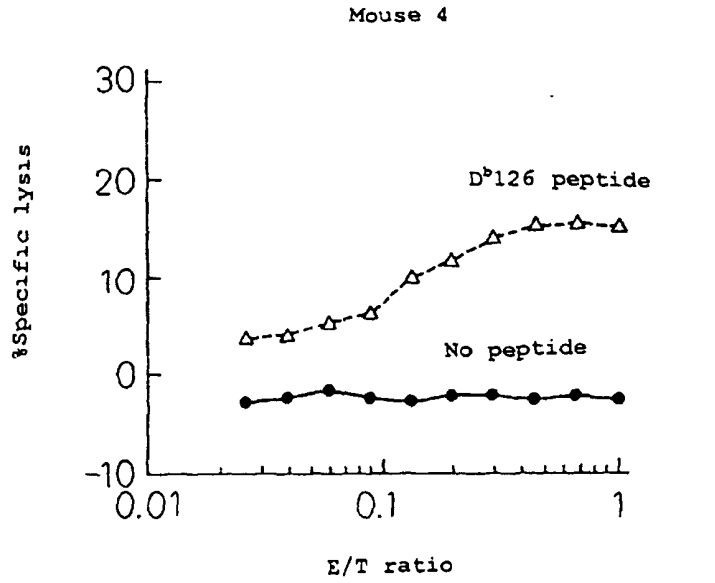

Mouse 4
%Specific lysis
30
20
10
0
-10
0.01
0.1
1
E/T ratio
D^b126 peptide
No peptide

Fig. 4

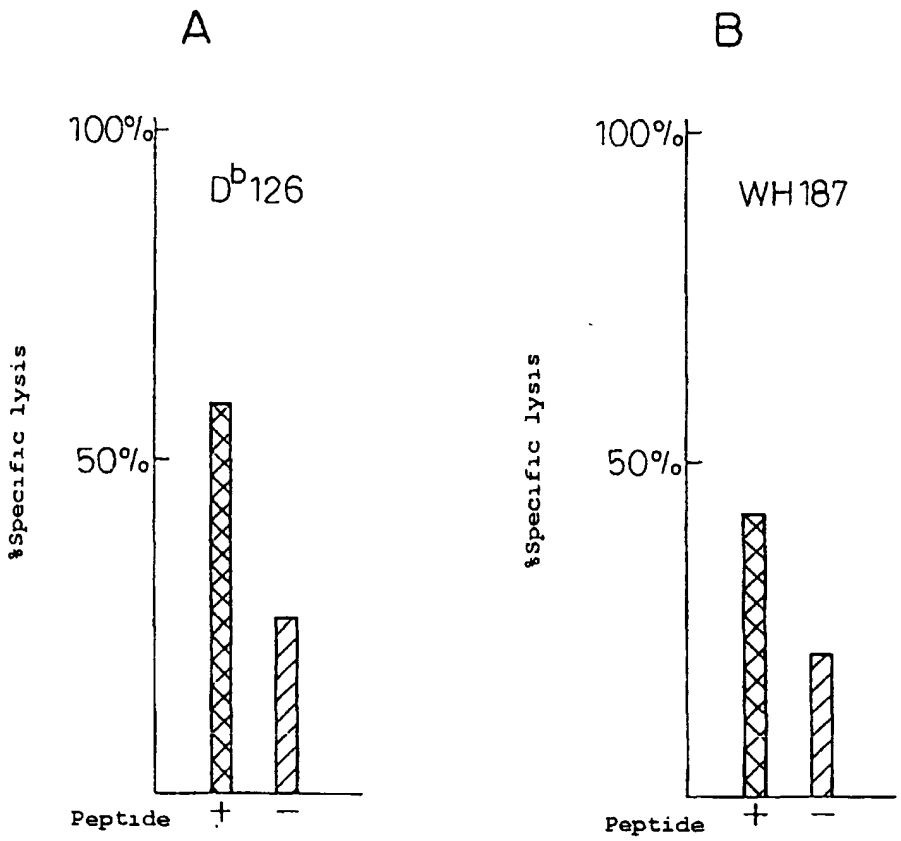
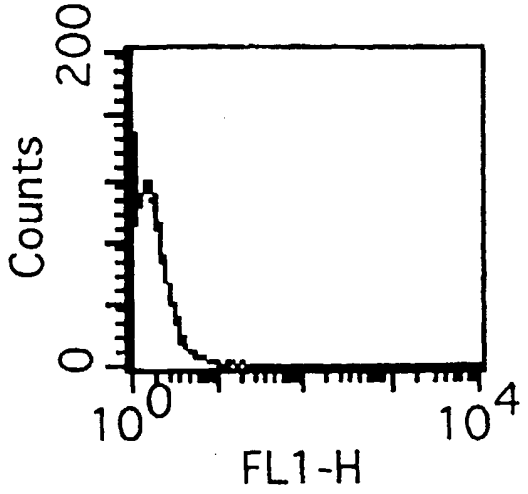

A
100%
50%
D^b126
%Specific lysis
Peptide
+
−
B
100%
50%
WH187
%Specific lysis
Peptide
+
−

Fig.5

CD19



CD3

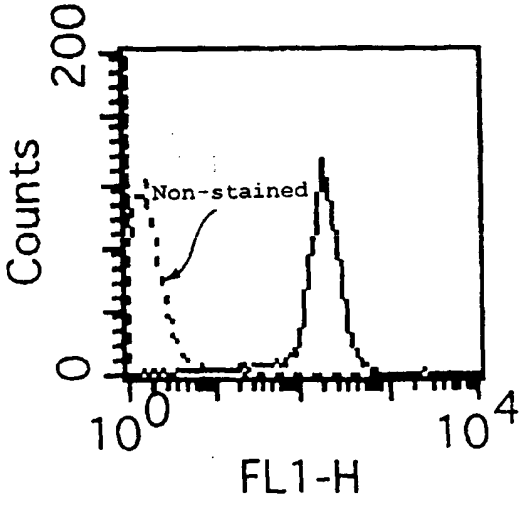
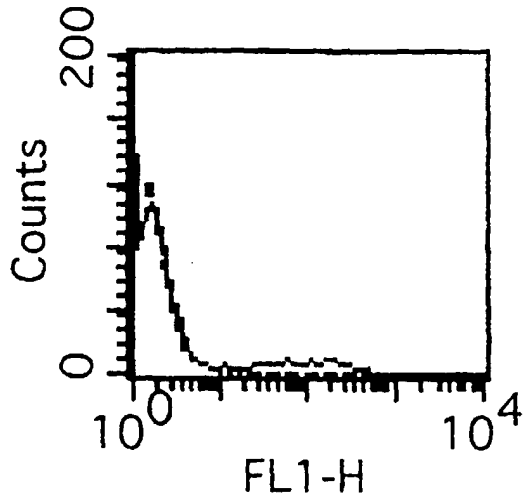


Fig.6

CD4


200
Counts
0
10^0
10^4
FL1-H

CD8

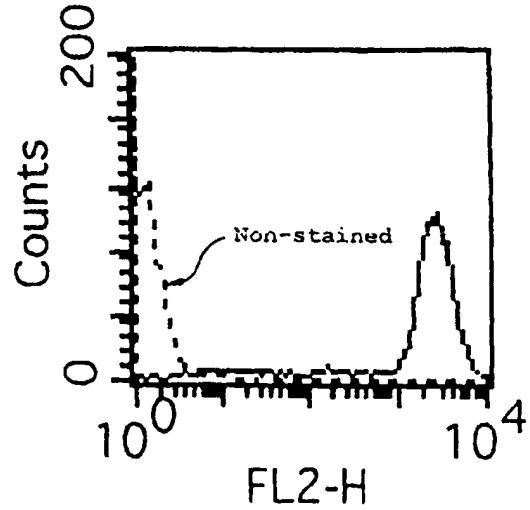

200
Counts
0
Non-stained
10^0
10^4
FL2-H

Fig.7

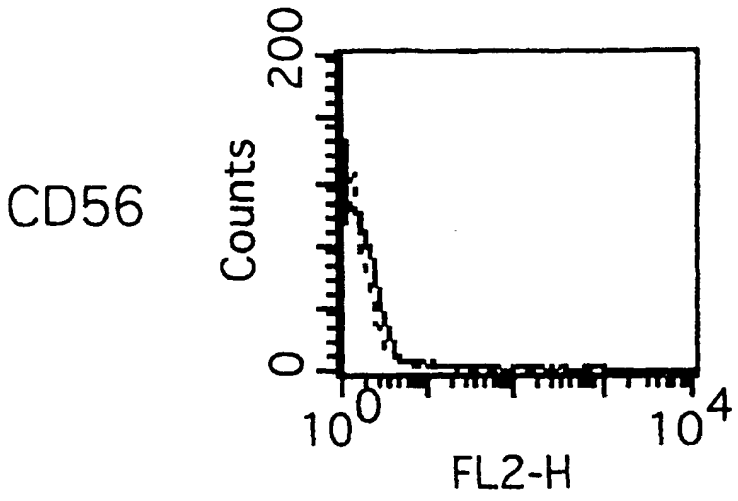
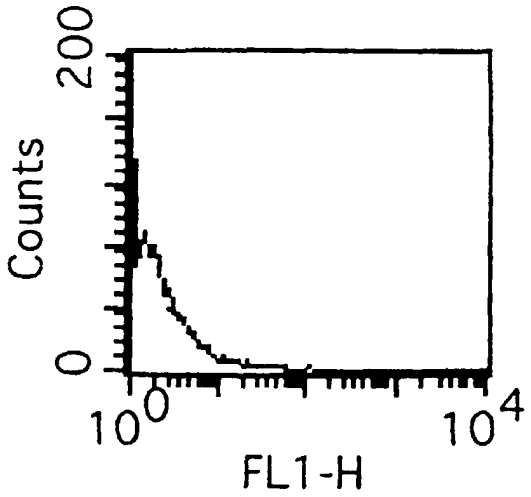


Fig.8

CD19



CD3

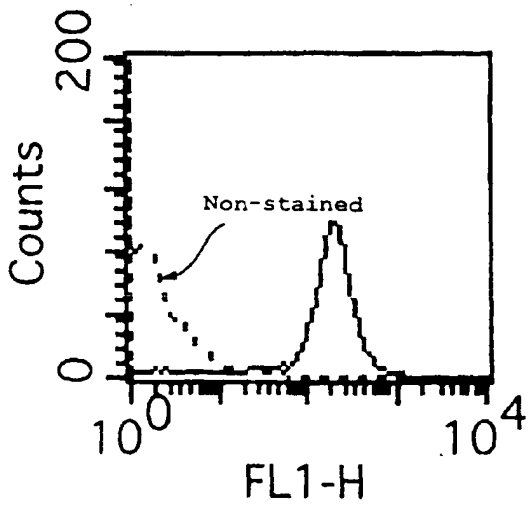
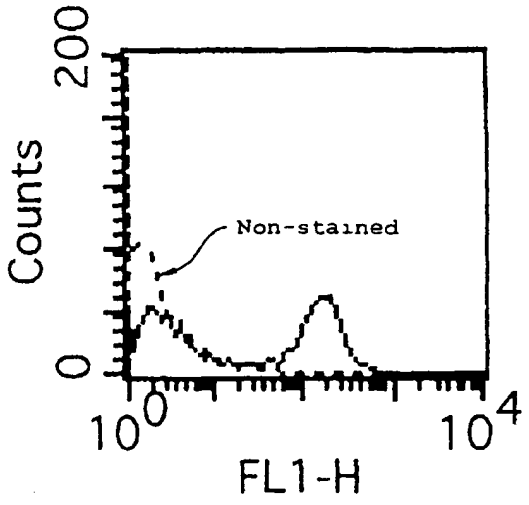


Fig.9

CD4


200
Counts
0
Non-stained
10^0
10^4
FL1-H

CD8

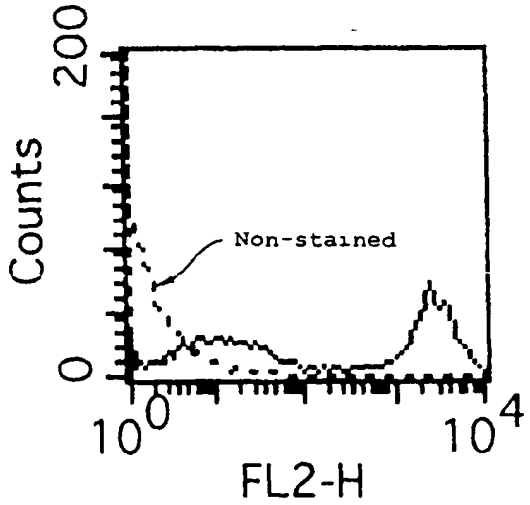

200
Counts
0
Non-stained
10^0
10^4
FL2-H

Fig.10

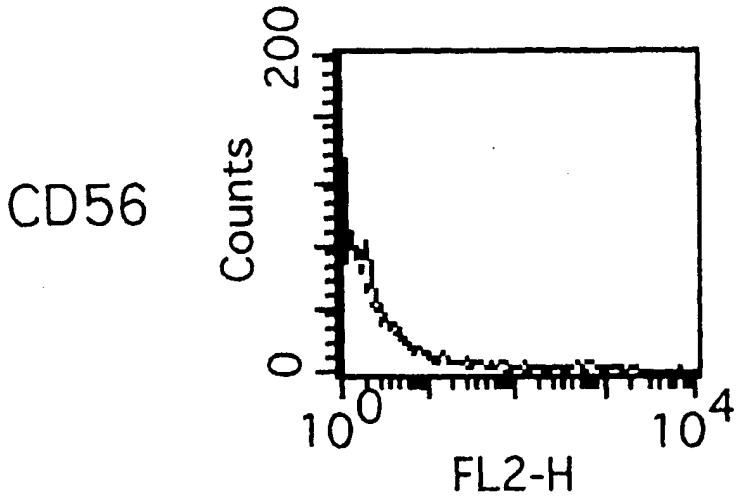


Fig.11

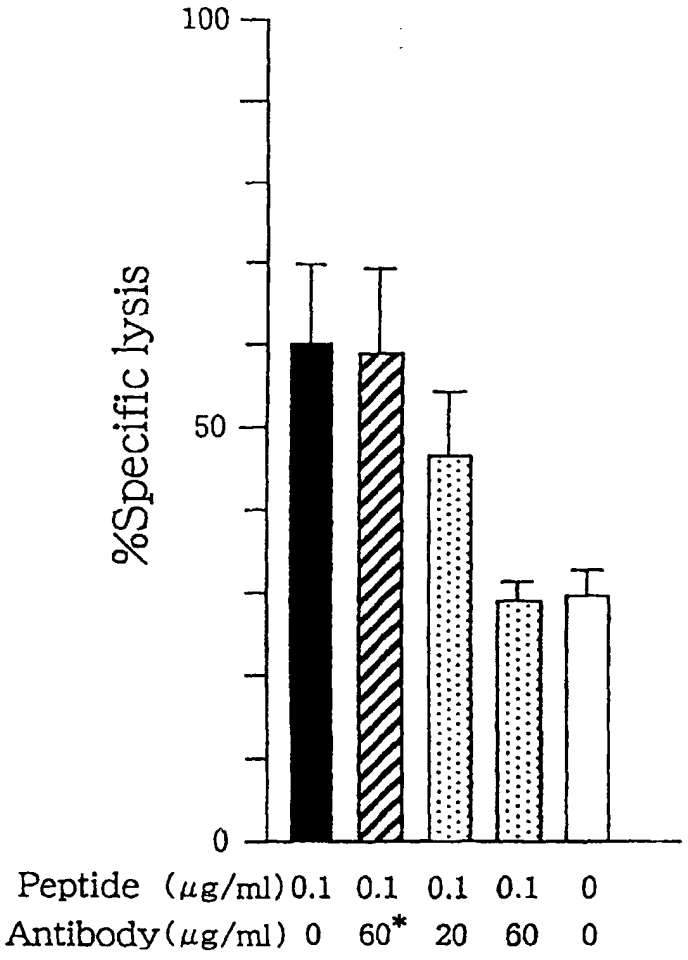

100
50
0
%Specific lysis
Peptide (μg/ml) 0.1 0.1 0.1 0.1 0
Antibody (μg/ml) 0 60* 20 60 0

Fig.12

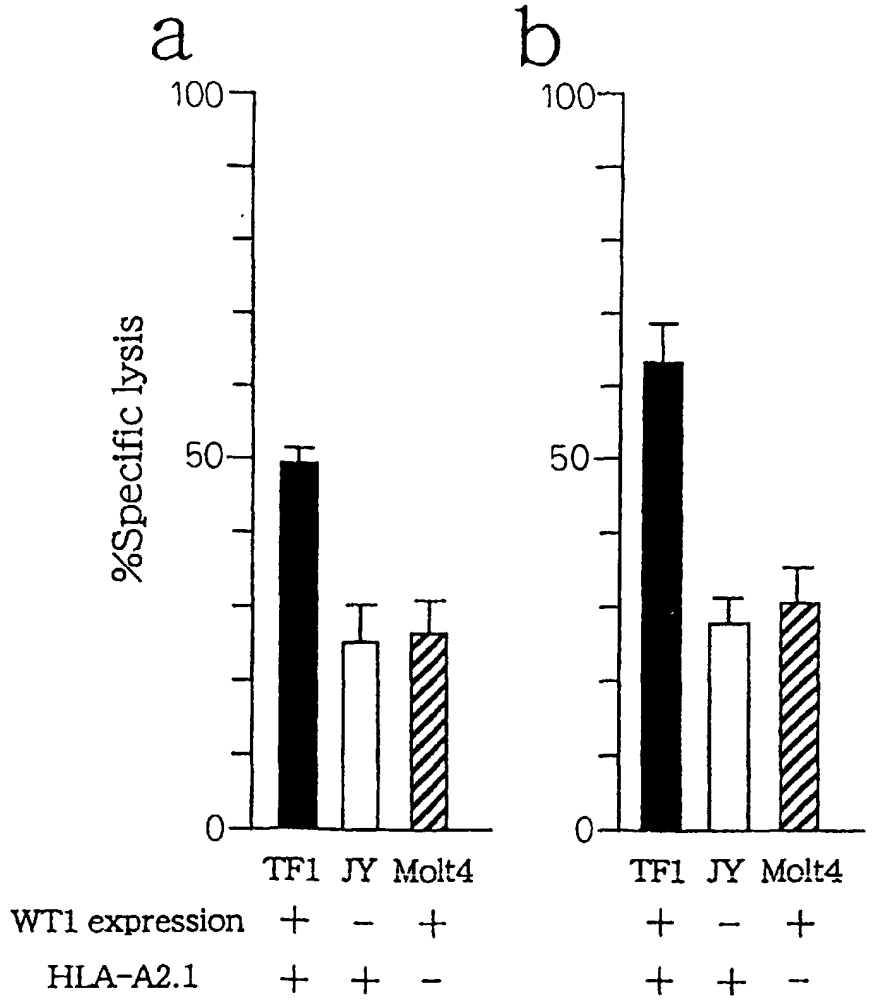

a
b
100
50
0
%Specific lysis
TF1
JY
Molt4
WT1 expression
HLA-A2.1
+
−

Fig.13

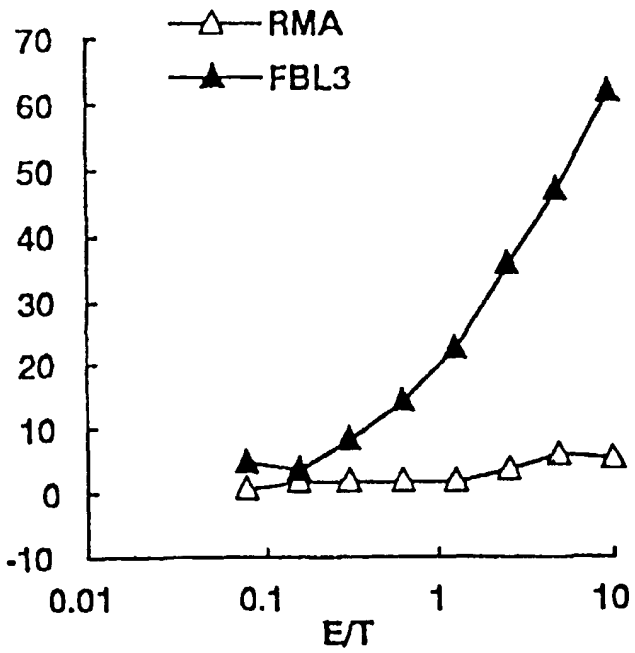

RMA
FBL3
70
60
50
40
30
20
10
0
-10
0.01
0.1
1
10
E/T

Fig.14

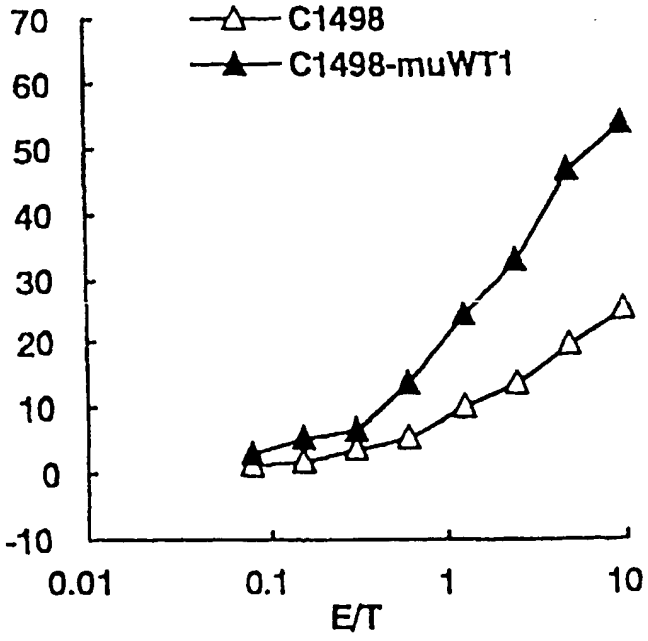

C1498
C1498-muWT1
70
60
50
40
30
20
10
0
-10
0.01
0.1
1
10
E/T

Fig.15

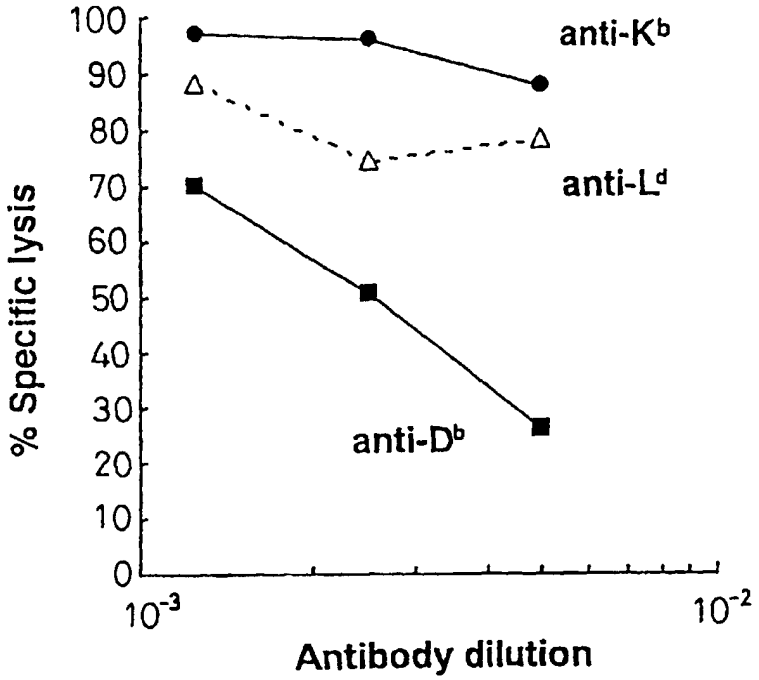

100
90
80
70
60
50
40
30
20
10
0
% Specific lysis
anti-K^b
anti-L^d
anti-D^b
10^-3
10^-2
Antibody dilution

Fig.16

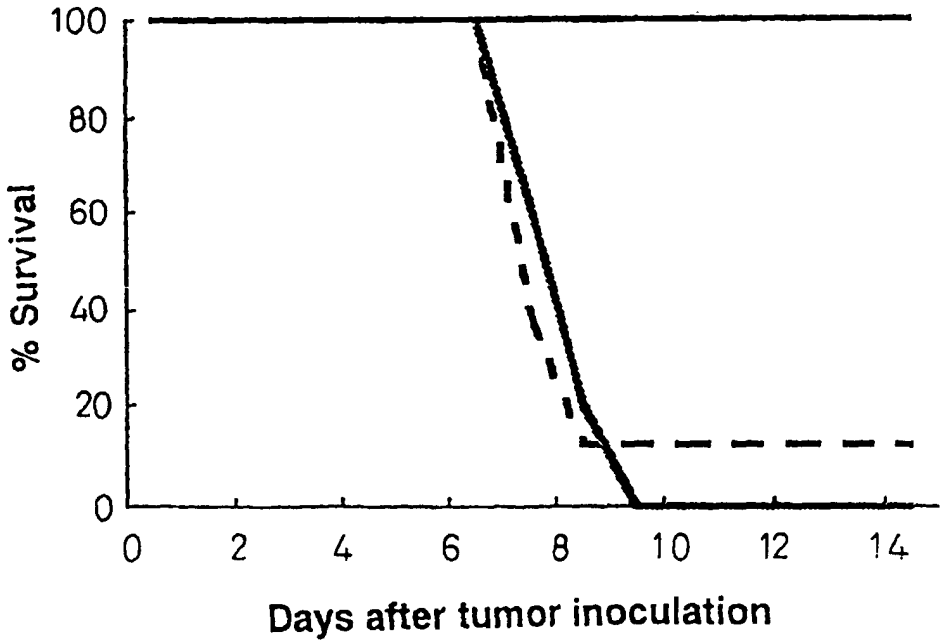

100
80
60
40
20
0
% Survival
0
2
4
6
8
10
12
14
Days after tumor inoculation

Fig.17

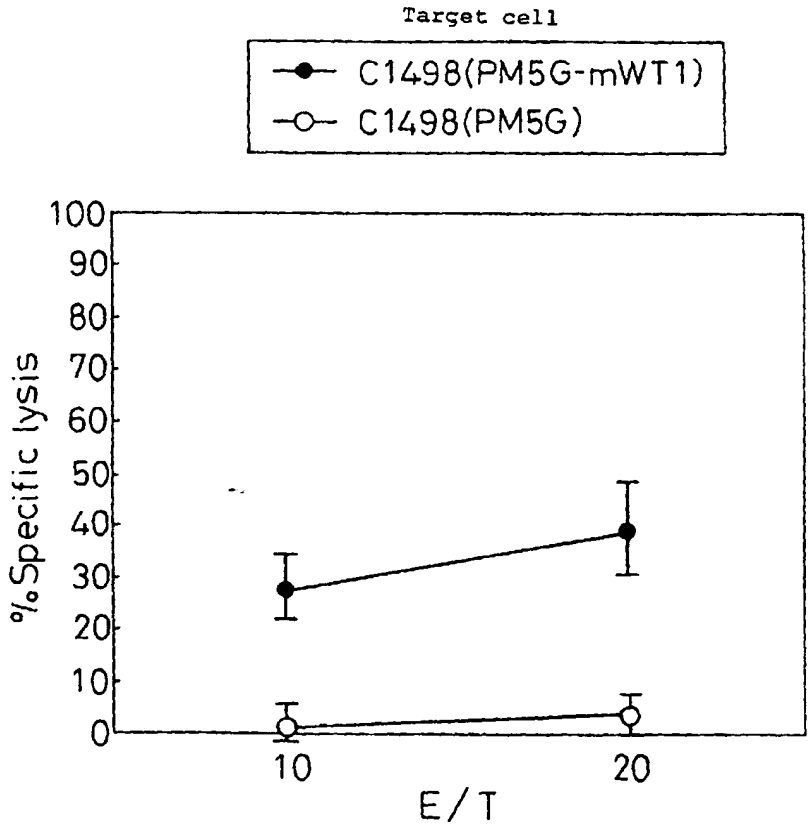
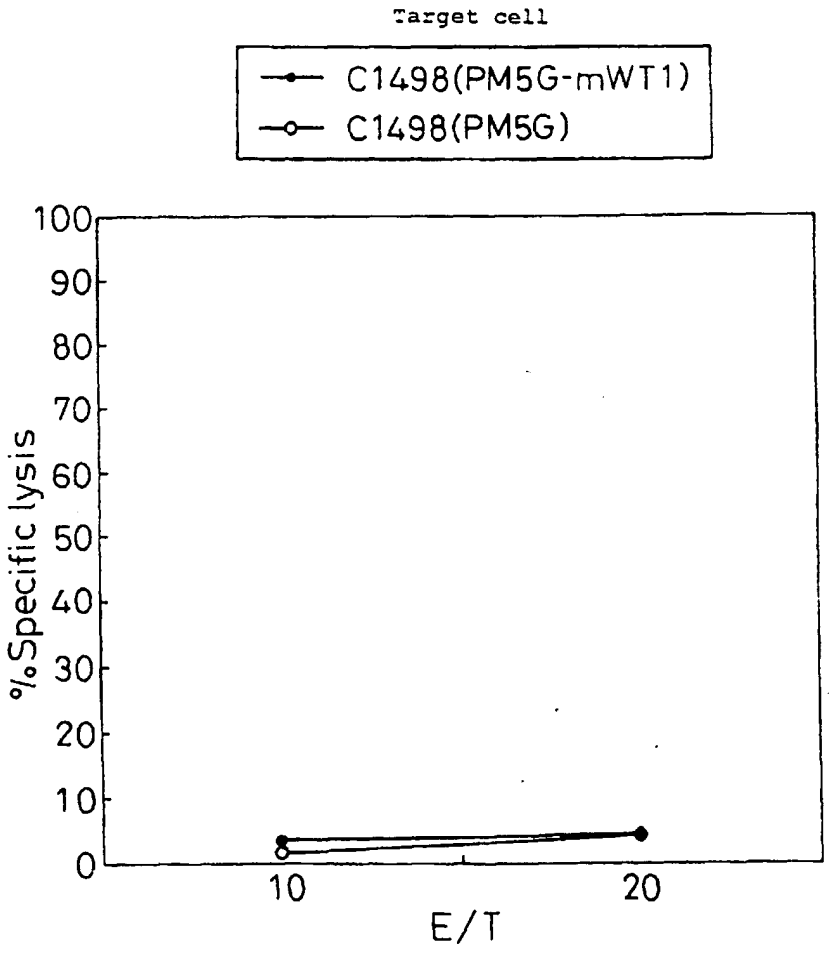

Target cell
C1498(PM5G-mWT1)
C1498(PM5G)
100
90
80
70
60
50
40
30
20
10
0
% Specific lysis
10
20
E/T

Fig.18


Target cell
C1498(PM5G-mWT1)
C1498(PM5G)
100
90
80
70
60
50
40
30
20
10
0
%Specific lysis
10
20
E/T

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/04130

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C07K14/82, C07K7/00, C12N15/00, A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C07K14/82, C07K7/00, C12N15/00, A61K39/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI (DIALOG), BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular and Cellular Biology, Vol. 11, No. 3 (1991) Alan J. Buckler, et al., "Isolation, Characterization, and Expression of the Murine Wilms' Tumor Gene (WT1) During Kidney Development", p.1707-1712	1-7
Y	JP, 9-104629, A (Chuzo Kishimoto, Haruo Sugiyama), 22 April, 1997 (22. 04. 97), Full text ; Figs. 1 to 10 & WO, 9638176, A & EP, 841068, A & AU, 9657796, A	1-6
Y	Current Opinion in Immunology, Vol. 5, No. 5 (1993) Pardoll DM, "New strategie for enhancing the immunogenicity of tumors", p.719-725	1-6
Y	Current Opinion in Immunology, Vol. 5, No. 5 (1993) Melief CJ, et al., "Potential immunogenicity of oncogenea and tumor suppressor gene products.", p.709-713	1-6

[x] Further documents are listed in the continuation of Box C. [] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October, 1999 (14. 10. 99)	2 November, 1999 (02. 11. 99)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/04130

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Immunogenetics, Vol. 41, No. 4 (1995) Rammensee HG, et al., "MHC ligands and peptide motifs: first listing.", p.178-228	1-6
PY	Proceeding of the American Association for Cancer Research Annual Meeting, Vo. 40 (March, 1999) Gaiger A, et al., "WT1: A new leukemia and cancer antigen.", p.424	7

Form PCT/ISA/210 (continuation of second sheet) (July 1992)